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<b>(54) Title:</b> A METASTASIS SUPPRESSOR GENE  <b>(57) Abstract</b>  A new human metastasis-suppressor gene was cloned using subtractive hybridization comparing metastatic and nonmetastatic cells. Loss of expression correlates with metastatic potential in human cells, the expression of the gene results in significant suppression of metastasis in athymic nude mice. A cDNA was isolated and the gene was designated <i>KiSS-1</i> and mapped to chromosome (1). The predicted <i>KiSS-1</i> protein has a proline-rich region with homology to SH3 binding domain which predicts a mechanism for metastasis suppression. <i>KiSS-1</i> and its products are useful as a marker for staging melanomas in a clinical setting, for diagnosis and for therapy.		

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## A METASTASIS SUPPRESSOR GENE

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This application claims priority from U.S.  
10 Provisional Application 60/005,080 filed October 11, 1995.

A cDNA molecule from the *KiSS-1* gene has a novel nucleotide sequence and encodes a protein that suppresses metastasis in melanoma cancers. The gene is useful as a  
15 marker to distinguish metastatic from non-metastatic cells, a characteristic forming the basis of diagnostic tests, and to provide criteria for disease staging, and planning therapeutic strategies. The *KiSS-1* nucleotide sequence is a candidate for use in gene therapy, and  
20 pharmaceutical compositions include the gene product of *KiSS-1*.

Metastasis, the spread of tumor cells from a primary tumor to form discontinuous tumors at nearby or distant sites is the major life-threatening complication to  
25 cancer cure. Metastasis is responsible for the majority of cancer-related deaths. In order to improve treatments and in order to more accurately diagnose and stage cancer, a greater understanding of the metastatic process at the molecular level is required. New modalities of  
30 cancer therapy are needed particularly to treat metastatic cancer. However, dissecting the specific genetic events controlling metastasis has been complicated by three factors. First, metastasis involves multiple steps, suggesting involvement of multiple genes  
35 (Liotta, 1992; Nicolson, 1993; Radinsky, 1991). Second,

metastatic cells are highly aneuploid; therefore, identifying structurally altered genes or abnormally functioning genes over background genomic instability in tumors has proven extremely difficult. Third, the ability of assays to identify metastasis-associated genes has been hampered by tumor heterogeneity and technical limitations. Nonetheless, the current paradigm of human tumor progression proposes that a finite number of nonrandom chromosomal and genetic alterations are responsible for each stage of neoplastic evolution (Vogelstein, 1990), including metastasis (Liotta, 1992; Nicolson 1993; Radinsky, 1991). Yet, the identities of metastasis-regulatory genes remain elusive.

Metastasis is known to be controlled at two levels. First, dominantly acting metastasis genes drive conversion toward metastatic spread (e.g., mutant c-Ha-ras and TIAM-1, reviewed in Dear, 1990; Liotta and Steeg, 1991; Liotta, 1991). Secondly, loss of some genes predisposes cells to convert from benign to malignant (e.g., nm23). The latter types of genes are termed "metastasis-suppressor genes."

Neuroectodermal cancers such as neuroblastoma, small cell lung carcinoma, and cutaneous and ocular malignant melanomas are examples of cancers for which metastasis is a serious problem. Melanoma currently accounts for only 1-5% of all cancers, but its incidence has increased more than 4% annually since 1970 (Armstrong, 1994; Koh, 1991). As with most solid tumors, the major complication preventing cure of melanoma is metastasis (Frost, 1992). Neoplastic progression toward malignancy of cutaneous melanoma can be described by relatively discrete stages - melanocyte tumorigenesis precedes formation of benign nevi (atypical moles) which presumably become fully tumorigenic before expanding radially (radial growth phase) then invading vertically

(vertical growth phase) into the skin. Some cells then acquire the ability to metastasize. Despite some defined criteria, it is still not always possible to correctly stage and predict a primary tumor's propensity to metastasize. Less subjective criteria are needed.

Genetic linkage studies have implicated chromosomes 1 and 9 in the predisposition to cutaneous malignant melanoma in families with higher-than-average risk for developing the disease (Dracopoli, 1989; Fountain, 1990; Trent, 1991; Parmiter, 1988; Dracopoli, 1988). Structural alterations of chromosome 6 and 7, on the other hand, occur in progressing or late-stage cutaneous malignant melanomas (Fountain, 1990; Trent, 1991; Dracopoli, 1992; Kacker, 1990; Guan, 1992). Recent data have suggested that the responsible gene on chromosome 9 is the multiple tumor suppressor gene, MTS1, which encodes for the cyclin-dependent kinase CDKN2 on chromosome 9 (Kamb, 1994; Parker, 1994; Skolnick, 1994; Hussussian, 1994). Loss of heterozygosity, homozygous deletions and chromosome breaks on the short arm of chromosome 9 in the vicinity of MTS1 are frequently found in non-hereditary melanomas, and these abnormalities arise early in tumor progression, i.e., in nevi and primary tumors (Cowan, 1988). Clinical studies are currently underway evaluating the utility of MTS1 as a diagnostic/staging aid.

Chromosome transfer studies with human melanoma cells have provided functional evidence for both tumor-suppressor and metastasis-suppressor genes on chromosome 6. Microcell-mediated chromosome transfer (MMCT) of chromosome 6 into UACC-903 and UACC-091 human malignant melanoma cell lines resulted in nontumorigenic hybrids (Trent, 1990), suggesting that chromosome 6 contains a melanoma tumor-suppressor gene. SOD2 (which maps to 6q25) (Church, 1992) and encodes manganese superoxide

dismutase) is thought to be a melanoma tumor-suppressor since transfection into the UACC-903 blocked tumor formation (Church, 1993).

Complete tumor suppression following introduction of chromosome 6 into human melanoma cells was somewhat surprising since alterations of chromosome 6 are generally considered late, rather than early, changes in melanoma (Fountain, 1990; Parmiter, 1988; Dracopoli, 1992). Moreover, when chromosome 6 was introduced into the highly metastatic human malignant melanoma cell lines, C8161 and MelJuSo, the resulting hybrids were still tumorigenic despite apparently retaining the entire added chromosome (Welch, 1994; Miele, 1995). neo6/C8161 hybrids were completely suppressed for their ability to metastasize following intradermal, subcutaneous or intravenous inoculation. C8161 and all neo6/C8161 hybrids retain SOD2 and express MnSOD; therefore, MnSOD is not apparently responsible for tumor suppression in this model (Welch, 1994). Likewise, transfection of SOD2 into C8161 clones does not suppress tumorigenicity or metastasis (Miele and Welch, 1995).

#### **SUMMARY OF THE INVENTION**

Genes expressed in nonmetastatic cells, but not in their metastatic counterparts, are termed "metastasis suppressor genes." Further support for this classification is obtained from the ability of a particular gene or gene product to suppress metastasis in a model system when the gene is introduced into metastatic cells. The availability of paired cell lines -metastatic and non-metastatic with otherwise the same genetic background, allowed the analysis of differentially expressed genes. Using two techniques to identify the differentially expressed genes, differential

display(a PCR-based technique) and subtraction libraries, novel human metastatic suppressor genes have been found.

A specific gene *KiSS-1* was identified using subtractive hybridization comparing parental, metastatic melanomas with chromosome 6-melanoma hybrid cells. The gene maps to chromosome 1q32 - q41. It is likely to be regulated by gene(s) on chromosome 6. Candidate genes were identified by searching for a minimum 10-fold increase in mRNA expression in nonmetastatic chromosome 6-C8161 hybrids cells (neo6/C8161.1) compared to highly metastatic parental C8161 cells grown under similar conditions. A resulting oligonucleotide probe was used to screen Northern blots of Meljuso, C8161 and neo/Meljuso cell lines. The gene designated *KiSS-1* was found to be more highly expressed in neo6/ hybrids than metastatic parental cells. Further support for the role of the gene product was that transfection of a *KiSS-1* cDNA into human melanoma cells suppressed metastasis in athymic mice, a model for metastasis.

A full-length *KiSS-1* cDNA (about 0.784 kb) has a novel nucleotide sequence. (SEQ ID No: 1) Other sequences within the scope of the invention include truncated sequences and fragments of the full length sequence. (SEQ ID Nos: 7, 8, 9, 10) These fragments or truncations of the basic sequence (SEQ ID No: 1) with or without nucleotide sequences that flank the fragments and/or extend the truncated sequence, are also within the scope of the present invention.

Characteristics of a cDNA with SEQ ID No:1 include about 0.784 kb and an open reading frame (ORF) of 0.495 kb. The cDNA encodes a protein of a predicted size of about 18 kD molecular weight and about 164 amino acids in length. The cDNA predicts a predominantly hydrophilic, 164 amino acid protein with a polyproline rich domain indicative of an SH3 ligand and a putative protein kinase

C- $\alpha$  phosphorylation site. Transfection of a full-length *KiSS-1* cDNA into human malignant melanoma cell line C8161 suppressed metastasis in an expression-dependent manner. The region 85-97 (SEQ ID NO: 5) includes the active site  
5 necessary for metastasis suppression. Truncated proteins and fragments of proteins that are useful for diagnosis, staging and therapy include amino acid sequences including positions 1-78 (SEQ ID NO: 3), 79-164, (SEQ ID NO: 4), 85-97 (SEQ ID NO: 5) (including the active  
10 region), and 154-156 (SEQ ID NO: 6) of SEQ ID NO: 2. No major regions of protein homology were found in comparing the amino acid sequences of the present invention with sequences in the SwissProt database.

Northern blots comparing a panel of human melanoma  
15 cells revealed that *KiSS-1* mRNA expression occurred only in nonmetastatic melanoma cells. Expression of mRNA in normal heart, brain, liver, lung and skeletal muscle was undetectable by Northern blotting. Weak bands were found in kidney and pancreas, but highest expression was  
20 observed in placenta.

A nucleotide sequence of the *KiSS-1* gene is useful for diagnosis and staging of breast cancer and neuroectodermal cancers including melanomas, small cell carcinoma of the lung and neuroblastomas. The gene is a  
25 candidate for gene therapy protocol, e.g. gene replacement. The protein encoded by the *KiSS-1* cDNA is also suitable for diagnostic tests and planning treatment strategies, as well as for use in pharmaceutical compositions. Monoclonal and polyclonal antibodies  
30 directed to proteins or polypeptides having amino acid sequences as shown in SEQ ID NOS: 2, 3, 4, 5 and 6 respectively are useful to detect the sequences.

For staging of cutaneous melanoma, the progression from melanocyte tumorigenesis, to benign nevi (atypical  
35 moles), to full tumors, to the radial growth phase is



accompanied by expression of the metastasis suppressor gene. As the melanoma enters the vertical growth phase, there is a reduction of the expression of the metastasis suppressor gene. Malignancy is detected by lack of KiSS-1 expression.

For diagnosis of a metastatic cancer, a probe for the KiSS-1 gene or at least the active fragment thereof (SEQ ID NO: 5) is applied to a biological test sample such as a skin or tumor biopsy under conditions suitable for hybridization. The probe may be labeled for ease of detection if hybridization occurs.

Diagnostic methods also include detecting expression of a tumor suppressor gene or fragment thereof by detecting KiSS-1 in mRNA or its gene product or fragments or truncations thereof.

Nucleotide sequences that are useful as probes to detect by hybridization related sequences in other cancers, are components of recombinant expression systems. Recombinant expression systems are useful to produce the KiSS-1 gene product or a fragment or truncation thereof, for example, for use in preparation of pharmaceutical compositions or for gene therapy. Suitable recombinant express systems comprise regulatory elements that allow expression of the KiSS-1 gene or a fragment or truncation thereof, such as a nucleotide sequence in accordance with SEQ ID NOS: 1, 7, 8, 9 or 10.

For gene therapy, a nucleotide sequence in accordance with SEQ ID NOS: 1, 7, 9, 10 or a functional equivalent (suppresses metastasis) is combined with suitable promoters, enhancers and inducible elements. In some embodiments, liposomes are used as gene carriers. Other suitable means of gene transfer are particle bombardment methods via a "gene gun," and electrofection. Alternatively, molecules with amino acid sequences in

accordance with SEQ ID NOS: 2, 3, 4, 5, or 6 are combined with suitable diluents in pharmaceutical compositions and administered to a mammal intradermally or systemically.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the nucleotide sequence (SEQ ID NO: 1) of a cDNA from a gene designated *KiSS-1*.

FIG. 2 shows the predicted protein sequence of *KiSS-1*. "Proteins" and "polypeptides" are used  
10 interchangeably herein. Abbreviations for amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The *KiSS-1* gene product has a polyproline-rich  
15 domain and a putative protein kinase C- $\alpha$  phosphorylation site.

The amino acids 81-100 show the PXXP consensus SH3 binding motif.

## 20 DESCRIPTION OF THE PREFERRED EMBODIMENT

Several candidate metastasis-suppressor cDNAs were identified by subtraction hybridization and differential display comparing C8161 and neo6/C8161 cells (Lee, 1996). One clone, designated *KiSS-1*, was expressed only in  
25 nonmetastatic neo6/C8161 cells. Full-length *KiSS-1* was obtained by cDNA library screening using a neo6/ C8161.1  $\lambda$ ZAP-cDNA library ( $7.5 \times 10^6$  plaques). After third round screening, seven positive clones were isolated and identified as a same gene by restriction mapping and  
30 sequence analysis. The longest cDNA insert was sequenced on both strands by the dideoxy chain termination method with Sequenase version 2.0 (Amersham). The cDNA designation combines interim laboratory nomenclature for putative Suppressor Sequences with acknowledgment of the

gene's discovery in Hershey. *KiSS-1* cDNA sequence was submitted to GenBank as a novel gene with an accession number of U43527.

A northern blot analyses of *KiSS-1* was used to  
5 investigate:

A. Expression of *KiSS-1* mRNA was sought in cell clones derived from C8161, neo6/C8161, and neo6(del)(q21-q23)/C8161 cell clones. neo6/C8161 hybrids were prepared using MCH262A1.D6 as a microcell chromosome donor (Trent, 10 1990) and neo6(del)(q21-q23) hybrids were prepared using a related variant A9/6q<sup>-</sup>Cl.1.2 (Gualandi, 1994) as a microcell chromosome donor. Relative metastatic potentials are based upon both experimental (intravenous inoculation into lateral tail vein) and spontaneous  
15 (intradermal inoculation into dorsolateral flank) metastasis assays. Poly (A)<sup>+</sup>-enriched mRNA was isolated from 80-90% confluent cells using FastTrack mRNA isolation kit (Invitrogen). Poly(A)<sup>+</sup> mRNA (2.5 µg) was loaded and electrophoresed on 1% agarose gel containing  
20 2.2M formaldehyde at 78V for 3.5 hr. The RNA was transferred onto a nylon membrane using the Turboblotter<sup>TM</sup> system (Schleicher & Schuell) and fixed using a UV crosslinker (Stratagene). Full-length *KiSS-1* cDNA probe was radiolabeled using random priming kit (Amersham).  
25 Prehybridization was done in 48% formamide, 4.8X SSC (1X SSC; 0.15M NaCl, 0.015M sodium citrate), 20 mM Tris [pH 7.6], 1X Denhart's solution (0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 10% dextran sulfate, and 0.1% SDS. Hybridization was carried out in  
30 the same buffer plus herring sperm DNA (100:µg) for 24 hrs at 42°C. The membrane was rinsed twice with 2X SSC/0.1% SDS for 15 min at 42°C, followed by twice washing with 0.1X SSC/0.1% SDS for 15 min at 42°C. The rinsed membrane was exposed to X-ray film (Dupont-NEN). *KiSS-1*

was undetectable in any cell line possessing metastatic potential, whether low or high.

B. Expression of KiSS-1 mRNA was sought in human metastatic melanoma cell lines: C8161; C8161cl.8; C8161cl.9; nonmetastatic hybrid cell clone neo6/C8161.1; Meljuso; A375P; A375M; MeWo; MeWo-3S5; MeWo-70W; M24met; and OM431. Poly(A)<sup>+</sup> RNA was isolated from each cell culture using a Micro-FastTrack mRNA isolation kit (Invitrogen). All cell lines having metastatic potential did not express KiSS-1.

C. Expression of KiSS-1 mRNA was sought in normal human tissues. A multiple-tissue RNA blot (2 µg of poly(A)<sup>+</sup> RNA per lane) was purchased from Clontech. The blot was hybridized using full-length of KiSS-1 probe. Detection of KiSS-1 message (1.0 kb) in placenta was possible following overnight exposure. KiSS-1 mRNA was detectable in pancreas (0.8 kb) and kidney (0.9 kb) only after 3-7 day exposure at -70°C with two intensifying screens. Besides differential organ expression, these results suggest that alternative splice variants of KiSS-1 may exist in different cells.

A full-length of KiSS-1 cDNA was cloned into pcDNA3 (Invitrogen) in which transcription is driven by a human cytomegalovirus promoter. The resultant plasmid pcDNA3-KiSS-1 was transfected into C8161 cells using Lipofectin (GIBCO-BRL). Individual transfectants were isolated and cloned following growth in the neomycin analog G-418 (600 µg/ml). Poly(A)mRNA was isolated from each transfectant using Micro-FastTrack mRNA isolation kit (Invitrogen) and northern blot analysis was done. C8161neomix is an uncloned population of C8161 cells transfected with pcDNA3 vector only. Two different sized transcripts are observed. KiSS-1 (1.0 kb) is smaller than "exogenous" KiSS-1 (1.3 kb) due to vector sequences in the latter.

Lanes labeled P1 and P2 are uncloned populations of C8161 cells transfected with pcDNA3-KiSS-1 construct.

**KiSS-1 is not expressed in metastatic melanoma cells**

The expression pattern of KiSS-1 was confirmed by  
5 northern blot analysis using an extensive panel of cell lines derived from the C8161 melanoma with widely ranging metastatic potentials. KiSS-1 mRNA expression could not be detected in any metastatic melanoma cell line. Expression was not detectable, even in overexposed blots  
10 or by RT-PCR, in parental C8161 or two subclones, C8161cl.9 and C8161cl.8, representing the highest and lowest metastatic potential among clones, respectively. KiSS-1 mRNA expression was also undetectable in C8161cl.9 microcell hybrids containing human chromosome 6 bearing a  
15 complex deletion within the region 6q21-q23 which remained metastatic in nude mice.

Seven independently prepared hybrids that contained an intact copy of human chromosome 6 in C8161 expressed high levels of a 1.0 kb transcript of KiSS-1 mRNA. Equal  
20 loading of lanes was confirmed by measuring GAPDH expression. All neo6/C8161 hybrid clones failed to metastasize following intravenous [experimental metastasis assay (Welch, 1994)], subcutaneous or intradermal [spontaneous metastasis assay (Welch, 1994)]  
25 injection into 3-4 week old, female athymic nude mice. Therefore, KiSS-1 mRNA expression appeared to be a qualitative marker, i.e., exclusive to nonmetastatic C8161 cell populations.

Expression of KiSS-1 mRNA was evaluated by northern  
30 blotting of other, unrelated human metastatic melanoma cell lines. To test for the possibility that truncated forms of KiSS-1 may have existed, a full-length cDNA probe was used. The 1.0 Kb KiSS-1 transcript or bands of other sizes were not detected in any cells capable of  
35 metastasizing in athymic nude mice.

The expression of KiSS-1 mRNA in human heart, brain, placenta, liver, lung, skeletal muscle, kidney or pancreas was also examined by northern blot analyses. Abundant KiSS-1 transcript (1.0 kb) was found in placenta, with very weak expression in kidney (detectable only after exposure for three or more days using two intensifying screens). Upon longer exposure, different transcript sizes of KiSS-1 were detected in pancreas (0.8 Kb) and kidney (0.9 kb), suggesting that alternative splicing may take place in different tissues. Expression of KiSS-1 could also be detected in normal human melanocytes by RT-PCR.

KiSS-1 cDNA (FIG. 1) has a single open reading frame that encodes a protein of 164 amino acids with a predicted molecular mass of 18 kDa. The initiation codon and surrounding nucleotides fit the Kozak consensus and there is a consensus polyadenylation site downstream of the termination codon. In vitro transcription and translation of a full-length KiSS-1 cDNA resulted in a single band at 18 kDa. A search of the GenBank and European Molecular Biology Laboratory (EMBL) databases revealed no significant homology with known genes. Similar lack of homology was seen for protein sequences in the SwissBank database.

#### **KiSS-1 suppresses metastasis when transfected into C8161 human melanoma cells**

In order to demonstrate a functional basis for the correlation of KiSS-1 expression and lack of metastatic potential, a full-length KiSS-1 cDNA expression vector was transfected into the C8161 human malignant melanoma cell line. Full-length cDNA of KiSS-1 was subcloned into the pcDNA3 constitutive expression vector and transfected into C8161. The vector alone was transfected as a negative control. Several clones were randomly selected

and analyzed for expression of *KiSS-1* by RNA blotting. The *KiSS-1* transcript in transfectants is larger (1.3 kb) due to additional sequences in the expression vector. The *in vitro* growth rates of the selected clones were not significantly different compared to parental metastatic C8161 cell clones. Clones expressing differing levels of *KiSS-1* transcript were tested for the metastatic ability in athymic nude mice using the experimental and spontaneous metastasis assays.

10 In the spontaneous metastasis assay, which measures the ability of cells injected intradermally or subcutaneously into the dorsolateral flank to metastasize to distant sites (Welch, 1991; 1983), *KiSS-1* transfectant clones 2, 3 and 9 were less able to colonize lung or  
15 regional lymph nodes than concomitantly injected C8161 cells (Table 1). Differences in metastatic potential could not be explained by slower *in vivo* primary tumor growth rate since several more metastatic subclones of C8161 grow more slowly than *KiSS-1* transfectants (Welch,  
20 1994). Parental C8161 cells yielded an average of 50 lung metastases per mouse and every mouse had regional lymph node metastases. In contrast, transfectant clone *KiSS-1cl.2*, which highly expressed *KiSS-1* RNA, produced an average of only 1 metastasis per mouse. None of the  
25 mice had more than 3 metastases and only two had lymph node metastases. *KiSS-1cl.3*, produced a mean of 3 metastases per mouse (all mice yielded less than 6 metastasis) and slightly higher incidences of lymph node metastases. Similar results were obtained following  
30 direct inoculation into the lateral tail veins of athymic nude mice (experimental metastasis assay, Table 2).

**The predicted KiSS-1 protein is an SH3 ligand or is phosphorylated by PKC- $\alpha$**

The predicted protein sequence of KiSS-1 cDNA was analyzed and the following homologies were found: a putative protein kinase C- $\alpha$  phosphorylation site (single-letter amino acid code, SMR corresponding to amino acid positions 154 to 156,) and a proline-rich region with five overlapping minimal SH3 binding domains (Cohen, 1995; Alexandropoulos, 1995). (PXXP motif, FIG. 2, corresponding to amino acids 85-97 where X can be any amino acid). This suggests that KiSS-1 is a ligand for a protein possessing an SH3 domain and/or is a substrate for PKC- $\alpha$  phosphorylation. Additionally, there are four cysteines leaving the possibility for intra- or inter-molecular disulfide linkages. The N-terminal 70 amino acids contain seven serines and a tyrosine (Y60) as possible phosphorylation sites. There are also five threonines in the KiSS-1 molecule. There are no apparent transmembrane, glycosylation, nuclear translocation or domains indicative of KiSS-1 being a kinase. Although predominantly hydrophilic, it is unlikely that KiSS-1 is a secreted protein based upon the rules of Von Heijne (von Heijne, 1986).

The PXXP motif is a constant feature of all SH3 ligands. The presence of five PXXP sequences in a 13 amino acid stretch is further evidence that KiSS-1 is an SH3 ligand (Cohen, 1995; Alexandropoulos, 1995; Hennessey, 1991; Musacchio, 1994). Rules governing specificity of SH3 binding are currently being elucidated (Cohen, 1995; Alexandropoulos, 1995; Joseph, 1995; Feng, 1994; Mayer, 1995; Rickles, 1995; Ishino, 1995); but, for the most part, they remain unknown. However, the putative KiSS-1 SH3 binding domain shares many features of the RLP-type (class I) ligand orientation.



SH3 domains are 50-70 amino acid modules present in a variety of intracellular proteins that mediate protein-protein interactions important for intracellular signaling and cytoskeletal organization (Koch, 1991; Musacchio, 1992; Pawson, 1992; Pawson, 1993; Feller, 1994; Nobes, 1995; Ridley, 1994; Cantley, 1991). Many of these interactions involving SH3 domains have been directly or indirectly associated with various steps in the metastatic cascade. Therefore, *KiSS-1* may suppress metastasis by regulating key signalling molecules important to one or more of these steps. *KiSS-1* transfectants did not display significantly influenced adhesion to the extracellular matrix components type-I collagen or fibronectin, nor was adhesion to a complex basement membrane-like mixture, Matrigel affected.

*KiSS-1* maps to chromosome 1q32-q41 by fluorescence *in situ* hybridization. Its mapping to another location within the genome leads to the conclusion that *KiSS-1* is regulated by the gene(s) on chromosome 6. *KiSS-1* is an important downstream effector of a gene(s) encoded on chromosome 6 which is consistent with metastasis suppression following introduction of chromosome 1 into MelJuSo melanoma cells. *KiSS-1* mRNA is not detected in metastatic neo6(del)(q21-q23)/C8161 hybrids. The latter observation can be explained by: (1) *KiSS-1* mutations, (2) *KiSS-1* deletions in subclones of C8161, or (3) mutation or deletion of regulatory gene(s) mapping to 6q21-q23. The latter explanation is consistent with high frequency loss of heterozygosity observed in late-stage melanomas (Trent, 1989; Guan, 1992; Milliken, 1991).

#### **Cell lines and culture conditions**

C8161 is an amelanotic human melanoma cell line that metastasizes widely following intradermal, subcutaneous or intravenous injection into athymic nude or SCID mice

Welch 1994; 1991). Subclones C8161cl.9 and C8161cl.8 were isolated from C8161 by limiting dilution and were chosen for these studies because they have the highest and lowest metastatic potentials, respectively (Welch, 1994). neo6/C8161.1, neo6/C8161.2 and neo6/C8161.3 (Welch, 1994) were derived from microcell-mediated chromosome transfer of a single copy of a neomycin-tagged human chromosome 6 using the MCH262A1.D6 donor cell line (Welch, 1994; Trent, 1990). Four additional, independently-derived neo6/C8161 hybrid cell clones (neo6/C8161.4, neo6/C8161.5b, neo6/C8161.6 and neo6/C8161.8) were subsequently developed. None of the neo6/C8161 hybrid cell clones were metastatic in athymic nude mice. neo6(del)(q21-q23) hybrid cell clones were prepared using a related chromosome 6 microcell donor variant A9/6q<sup>-</sup>cl.1.2 (Gualandi, 1994) and are metastatic in nude mice.

Cell line nomenclature has been chosen to identify the origin and nature of each cell line as unambiguously as possible. Single cell clones are preceded by a "." (e.g., C8161cl.9 is a single cell clone) and pooled, uncloned populations are identified by a "-" (e.g., C8161-KiSS-1-P1 is an uncloned population #1 of C8161 cells transfected with KiSS-1). Microcell hybrids are identified by the tagged chromosome followed by a "/" (e.g., neo6/C8161.3 is single cell clone 3 derived from a fusion with a neo-tagged human chromosome 6; neo6(del)(q21-q23)/C8161cl.8 is single cell clone 8 derived from a microcell fusion with a neo-tagged chromosome 6 containing deletion of the q21-q23 bands).

All cells were grown in DME-F12 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (cDME-F12). neo-resistant cells, neo6/C8161 and neo6(del)(q21-q23)/C8161 hybrids were maintained in cDME-F12 containing 500 µg/ml geneticin (G-418, GIBCO-BRL,

Gaithersburg, MD). Nearly confluent cultures (70-90%) were passaged in Corning (Oneata, NY) tissue culture dishes following detachment with a solution of calcium- and magnesium-free Dulbecco's phosphate buffered saline containing 2 mM EDTA at split ratios of 1:10 to 1:20. All cultures were routinely tested and found to be negative for *Mycoplasma spp.* infection using a PCR-based test kit (PanVera, Madison, WI).

10        **Subtractive hybridization**

Subtractive hybridization was done as described (Lee, 1991; Duguid, 1988; Schweinfest, 1990) with minor modifications. Briefly, mRNAs were isolated from 80-90% confluent cells using FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA). A cDNA library was constructed from neo6/C8161.1 cells using λZAP-cDNA Gigapack II Gold™ cloning kit (Stratagene, La Jolla, CA). Subtractive hybridization was carried out between the first strand cDNA from nonmetastatic neo6/C8161.1 cells and mRNA from metastatic parental C8161 cells. cDNA was synthesized from 1 µg poly(A)<sup>+</sup> enriched RNA from neo6/C8161.1 using oligo(dT) primers. Biotinylation of mRNA from parental C8161 cells was performed using Photobiotin™ labeling system (GIBCO-BRL) according to manufacturers instructions. To subtract, biotinylated mRNA (20 µg) was mixed with cDNA (1 µg) from neo6/C8161.1 and ethanol precipitated. The pellet was resuspended in DEPC-treated water (20.5 µl) then added to 2X hybridization buffer [80% formamide, 100 mM hydroxyethylpiperazine ethane sulfonic acid (pH 7.5), 2% sodium dodecyl sulfate, 22.5 µl]. This mixture was boiled for 2 min, chilled on ice for 5 min followed by addition of NaCl (5 M, 2µl). Hybridization was performed at 42°C for 48 hr. The hybridization mixture was added to streptavidin (25 µg, Sigma) and incubated at room

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temperature for 5 min before extraction with phenol:chloroform: isoamylalcohol (25:24:1). The organic phase was twice back-extracted with 50  $\mu$ l streptavidin-binding buffer [100 mM Tris-HCl pH 8.0; 1 mM EDTA, 500 mM NaCl]. Then the aqueous phases were pooled. Unhybridized cDNA target was ethanol precipitated. This subtracted cDNA was used as a probe for the neo6/C8161.1 cDNA library screening following random primer labeling (Amersham). Positive clones were isolated and used for evaluation of expression in northern blot analysis.

**Screening candidate cDNAs and isolation of full-length KiSS-1**

Candidate cDNAs were initially evaluated for differential expression by Northern blotting using poly(A)<sup>+</sup> enriched mRNA from C8161 and neo6/C8161.1 cells. If expression was greater in neo6/C8161.1 cells by at least 10-fold, the same cDNA was used to probe a more extensive blot containing RNAs from a panel of cell lines with differing metastatic potentials. If the pattern of expression correlated with metastatic potential, full-length cDNAs were isolated and further characterized.

Full-length KiSS-1 was obtained from a  $\lambda$ ZAP-cDNA library ( $7.5 \times 10^6$  plaques) prepared from neo6/C8161.1 cells. After third round screening, seven positive clones were isolated and identified as a same gene by restriction mapping and sequence analysis. Library screening was done using a 0.5 kb partial KiSS-1 probe obtained from the subtractive hybridization. The longest cDNA insert was sequenced on both strands by the dideoxy chain termination method with Sequenase version 2.0 (Amersham).

### Northern blot analysis

For Northern blot analysis, poly(A)<sup>+</sup> enriched mRNA was isolated from 80-90% confluent cells using FastTrack mRNA isolation kit (Invitrogen). Poly(A)<sup>+</sup> mRNA (2.5 µg) was loaded and electrophoresed on 1% agarose gel containing 2.2M formaldehyde at 78V for 3.5 hr. RNA was transferred onto a nylon membrane using the Turboblott<sup>TM</sup> system (Schleicher & Schuell) and fixed by UV crosslinking (Stratagene). Full-length KiSS-1 cDNA probe was radiolabeled using random priming (Amersham). Prehybridization was done in 48% formamide, 4.8X SSC (1X SSC; 0.15M NaCl, 0.015M sodium citrate), 20 mM Tris [pH 7.6], 1X Denhart's solution (0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 10% dextran sulfate, and 0.1% SDS. Hybridization was carried out in the same buffer plus herring sperm DNA (100: µg) for 24 hrs at 42°C. The membrane was rinsed twice with 2X SSC/0.1% SDS for 15 min at 42°C, followed by twice washing with 0.1X SSC/0.1% SDS for 15 min at 42°C. The rinsed membrane was exposed to X-ray film (Dupont-NEN) for desired intensity.

Expression of KiSS-1 in normal human tissues was evaluated using a mutiple-tissue RNA blot (2 µg of poly (A) RNA per lane) purchased from Clontech. The blot was hybridized using full-length KiSS-1 probe as above. Expression of KiSS-1 in normal human melanocytes (Clonetics) was determined by RT-PCR.

### Transfections and metastasis assays

Full-length of KiSS-1 cDNA was cloned into the pcDNA3 expression vector (Invitrogen) in which transcription is driven by human cytomegalovirus promoter. The resultant plasmid pcDNA3-KiSS-1 was transfected into C8161 cells using Lipofectin (GIBCO-BRL). Individual transfectants were isolated and cloned

following growth in cDME-F12 containing G-418 (500  $\mu\text{g/ml}$ ). Poly(A)<sup>+</sup> mRNA was isolated from each transfectant using Micro-FastTrack mRNA isolation kit (Invitrogen) and northern blot analysis was done as described above.

- 5 C8161neomix was isolated as a control, uncloned population of C8161 cells transfected with pcDNA3 vector only.

Transfectants were evaluated for growth *in vitro*. Cells ( $2 \times 10^4$ ) were inoculated into 24-well tissue  
10 culture plates (Corning) in cDME-F12 medium. After 24, 48, 72, and 96 hr, cells were trypsinized and counted using a hemacytometer.

For spontaneous metastasis assays, cells ( $1 \times 10^6$ ) were injected intradermally into the dorsolateral flank  
15 of athymic nude mice (4-12 mice per group). Three- to four-week old female mice (Harlan Sprague Dawley, Madison, WI) were used for these studies. Animals were maintained under the guidelines of the National Institutes of Health and The Pennsylvania State  
20 University College of Medicine. All protocols were approved by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. Tumor size was measured weekly by taking orthogonal measurements and expressed as mean tumor diameter. Mean  
25 tumor diameter was calculated as described (Welch, 1994; Claas, 1996). After the mean tumor diameter reached 1.5-2.0 cm, mice were necropsied, and visible metastases were counted (Welch 1991; 1983).

In the C8161 human melanoma model, rank order of  
30 spontaneous and experimental metastasis assays for multiple cell variants are equivalent (Welch, 1991). For experimental metastasis assays, cells ( $2 \times 10^5$  to  $3 \times 10^5$ ) suspended in ice-cold Hank's balanced salt solution (0.2 ml) were injected into the lateral tail vein of 3-4 wk  
35 old, female athymic nude mice. After 4 wk, mice were

killed by cervical dislocation and metastatic lesions were scored as described (Welch, 1991; 1983). Quantification of metastasis formation is identical regardless of metastasis assay employed. Briefly, lungs  
5 from each mouse were removed and fixed in a mixture of formalin and Bouin's fixative (5:1 v/v) and examined under a dissecting stereomicroscope (Welch, 1983). Unless otherwise noted, all other tissues were examined and found to be free of metastases.

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### **Statistical analysis**

The number of lung metastases was compared in KiSS-1 transfectants and parental C8161 cells. For experimental metastasis assays, One-way ANOVA followed by Tukey's  
15 Honestly Significant Difference post-test was used. For spontaneous metastasis assays, a Kruskal-Wallis ANOVA of ranks procedure was used. Calculations were performed using SigmaStat statistical analysis software (Jandel Scientific). Statistical significance was defined as  
20  $P \leq 0.05$ .

### **Preparation of microcell hybrids**

MCH262A1.D6 or MCH556.1 were used to prepare microcells according to standard methods (Saxon 1987;  
25 Sanford, 1987). Briefly, microcell hybrids are made by treating proliferating (80% confluent) donor cells with colcemid (0.15  $\mu\text{g/ml}$ ) for 48 h. Chromosomes recondense and form micronuclei because spindle formation is blocked. Each micronucleus contains one or more  
30 chromosomes which are then enucleated by treatment with cytochalasin B (2  $\mu\text{g/ml}$ ) and centrifugation. The resulting microcells are sequentially filtered through 8, 5, and 3  $\mu\text{m}$  polycarbonate filters to obtain a population containing mostly single chromosomes. The microcells are

fused using polyethylene glycol to a semi-confluent recipient C8161 cell culture. After fusion and recovery (24 h), cells are grown under selective conditions, i.e., cDME-F12 containing G-418 (600 µg/ml). Only recipient  
5 cells that contain the neo-tagged chromosome will survive. Individual colonies were isolated.

**PCR-RFLP Verification of Chromosome 6 in C8161 and neo6 hybrid cell lines and tumors**

10 Genomic DNA from cell lines, tumors, lung metastases and donor cell line was isolated (Sambrook, 1989) and digested with EcoRI. The GT primer for the more than polymorphic (CA)<sub>n</sub> repeat markers radiolabeled with γ [<sup>32</sup>P]CTP at 37°C for 45 mins by T4 polynucleotide kinase.  
15 DNA (50 ng) was added to a 30 µl PCR reaction mix and amplified for 28 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min). An apparently intact added chromosome was found in all cell lines, tumors and metastases evaluated.

20 **Isolation of poly (A) RNA**

Poly(A)<sup>+</sup> RNA used for Northern blot analysis and cDNA library construction was isolated from 80-90% confluent cells using Fastrack™ mRNA isolation kit (Invitrogen, San Diego, CA).

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**Construction of cDNA library**

A cDNA library from neo6/C8161.1 cells was constructed using λZAP-cDNA Gigapack II Gold™ cloning kit (Stratagene, La Jolla, CA). The first strand cDNA  
30 was synthesized using a hybrid oligo(dT) linker-primer, containing an XhoI restriction site, from 5 µg poly(A)<sup>+</sup> RNA isolated from neo6/C8161.1 cells. After second strand DNA was synthesized, an EcoRI adapter was ligated,



and cDNA fragments were digested with XhoI, and size-fractionated by Sephacryl S-400 spin column. Fractions containing cDNA fragments larger than 500 bp were pooled and used for ligation into the EcoRI-XhoI site of UniZap II arms. Ligation mixtures were packaged *in vitro* with Gigapack Gold extracts, and the resulting phage library was titered and amplified on the *E. coli* XL1 blue cells. After a single round of amplification, the phage library titer was  $5 \times 10^{10}$  plaque-forming unit (pfu)/ml.

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### Screening of cDNA library

Recombinant plaques were plated a density of 10,000 pfu/150 mm plate. Lifting of plaque onto a membrane (Colony/Plaque Screen<sup>TM</sup> Hybridization Membrane, Dupont-NEN, Boston, MA) was performed according to the manufacturer's protocol. Prehybridization of the immobilized plaques was carried out for 1 hr at 42°C in 48% formamide/4.8X SSC<sup>1</sup>/20 mM Tris (pH 7.6)/1X Denhart's solution/10% dextran sulfate/0.1% SDS. Hybridization was carried out for 16-24 hrs at 42°C in prehybridization mix plus 100 µg/ml herring sperm DNA, plus radiolabeled cDNA probe. After full-length KiSS-1 had been obtained, screening was performed using recombinant plaques plated at a density of 50,000 pfu/150 mm plate.

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### DNA sequence analysis

The plasmid DNA was isolated using Wizard<sup>TM</sup> miniprep kit (Promega, Madison, WI). DNA sequences were determined by double stranded DNA sequencing using the dideoxy chain termination method with Sequenase version 2.0 (Amersham). Computer-assisted DNA sequence analysis

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<sup>1</sup>1XSSC = 0.15M sodium citrate Denhardt's solution = 0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone.

was performed with FASTA program to search sequence identity.

**A Method for Diagnosing  
Metastasis in a Biological Sample**

To diagnose metastasis in a subject, a biological sample is obtained from the subject. Suitable samples include skin and tumor biopsies. The sample is processed by methods known to those of skill in the art, e.g. tissues are sectioned, fixed and mounted, so that histochemical staining or in situ hybridization is facilitated. Antibodies to polypeptides encoded by the KiSS-1 gene are prepared and labeled for detection in the biological sample. Suitable polypeptides include those having amino acid sequences in accordance with SEQ ID NOS: 2, 3, 4, 5 of the present invention. Suitable antibodies are preferably monoclonal antibodies. Riboprobes (antisense molecules) directed to nucleotide sequences as shown in SEQ ID NOS: 1, 7, 8, 9, and possibly 10, are used for in situ hybridization to detect the expression of the KiSS-1 gene (Ausubel, 1990). Suitable labels include S-35, biotin -substituted nucleotides, digoxigenin.

The three possible types of outcomes for either the assays for the polypeptide products of KiSS-1 expression, or for mRNA complementary to the KiSS-1 gene, and the interpretation of those outcomes are: 1. If there is evidence of KiSS-1 expression, the biological sample is classified as non-metastatic; 2. If there is no evidence of KiSS-1 expression, the biological sample is classified as metastatic; and 3. If either there is evidence of KiSS-1 expression in some cells or portions of a biological sample, but not in other cells or portions of the tissue, a decision on whether to classify the sample as metastatic or non-metastatic requires a

comparison to percentages ( or levels of expression in a fluid sample) of expression developed from normal control samples and from samples of the cancer in different stages. Staging of cancers is discussed in Hollev et  
5 el.(1991) and is cancer-type specific.

**EXAMPLES**

Examples are provided not for limitation of methods and compositions of the present invention, but for illustration.

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**EXAMPLE 1: Detection of KiSS-1 mRNA by Riboprobes to Stage Malignant Melanoma**

To supplement or refine staging for malignant melanoma, KiSS-1 gene expression was detected in 6 cell lines. The following cell lines were tested for KiSS-1 gene expression using RT-PCR (reverse transcriptase polymerase chain reaction). 1. A normal melanocyte cell line in which gene expression was demonstrated; 2. A cell line (WM35) derived from a melanoma in the radial growth phase in which gene expression was detected; 3. A cell line (WM793) derived from a melanoma in the early vertical growth phase gave inconclusive results of gene expression; 4. A metastatic melanoma cell line (WM115) in the vertical growth phase showed no evidence of gene expression; and 5. Two malignant melanoma cell lines (1205-LU and WM239A) showed no gene expression.

The conclusion from these studies was that there is correlation between gene KiSS-1 expression and clinical staging of melanoma. This correlation forms the basis for diagnosis and staging methods, that may assist therapeutic decisions. Because the gene expression is lost leading to metastasis, gene replacement is a viable clinical opportunity.

Table 1. *KiSS-1* suppresses spontaneous metastasis of C8161 human melanoma cells

Cell line	Relative <i>KiSS-1</i> expression	Lung Metastases			Extrapulmonary
		Incidence	Mean $\pm$ S.E.M.	Median (range)	Incidence
C8161	-	16/16	50 $\pm$ 25	9.5 (2,>200)	8/8
<i>KiSS-1</i> cl.2	+++	5/7	1.1 $\pm$ 0.4	1 (0, 3)	2/8
<i>KiSS-1</i> cl.3	+	6/7	2.7 $\pm$ 0.7	3 (0,6)	4/8
<i>KiSS-1</i> cl.9	++	0/7	0	0	2/7

Cells ( $1 \times 10^6$ ) were injected intradermally into the dorsolateral flank of 3-4 week old, female athymic nude mice (7-8 mice per group, Harlan Sprague Dawley). When mean tumor diameter (square root of the product of orthogonal measurements) reached 1.5-2.0 cm, mice were killed. All organs were examined for presence of metastases and many were confirmed by microscopic examination of hematoxylin- and eosin-stained paraffin-embedded sections (4-6  $\mu$ m). Macroscopic lung metastases were quantified after staining with in a mixture of formalin:Bouin's fixative (5:1) and counting with the aid of a dissecting microscope as previously described (Welch, 1991).

Table 2. *KiSS-1* suppresses experimental metastasis of C8161 human melanoma cells

Cell line	Relative <i>KiSS-1</i> Expression	Lung Metastases			
		Incidence	Mean $\pm$ S.E.M.	Median (range)	P-value
C8161	-	12/12	152 $\pm$ 25	>200 (2, >200)	
C8161cl.9	-	4/4	200 $\pm$ 41	>200 (98, >200)	NSD
C8161- neomix	-	4/4	160 $\pm$ 26	174 (93, >200)	NSD
<i>KiSS-1</i> cl.12	+	8/8	81 $\pm$ 31	40 (1, >200)	P<0.05
<i>KiSS-1</i> cl.3	+	16/16	82 $\pm$ 49	101 (2, >200)	P<0.05
<i>KiSS-1</i> cl.9	++	15/16	21 $\pm$ 13	24.5 (0, 80)	P<0.001
<i>KiSS-1</i> cl.10	+++	4/12	1 $\pm$ 1	0 (0, 4)	P<0.0001
<i>KiSS-1</i> cl.2	+++	9/16	0.9 $\pm$ 0.4	0.5 (0, 3)	P<0.001

Cells ( $3 \times 10^5$ ) were injected intravenously into the lateral tail vein of 3-4 week old, female athymic nude mice (7-8 mice per group, Harlan Sprague Dawley). Mice were killed 26 days post-inoculation. All organs were examined for presence of metastases and many were confirmed by microscopic examination of hematoxylin- and eosin-stained paraffin-embedded sections (4-6  $\mu$ m). Macroscopic lung metastases were quantified with the aid of a dissecting microscope after staining with a mixture of formalin:Bouin's mixture (5:1) as described (Welch, 1983; 1991). P-values were calculated by one-way ANOVA using Tukey's Honestly Significant Difference post-test. The values shown compare each group with parental C8161.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Welch, Danny R.  
Lee, Jeong-Hyung

10

(ii) TITLE OF INVENTION: A Gene Encoding For A Metastasis  
Suppressor Gene

(iii) NUMBER OF SEQUENCES: 10

15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Brinks, Hofer, Gilson & Lione  
(B) STREET: P.O. Box 10395  
(C) CITY: Chicago  
(D) STATE: IL  
(E) COUNTRY: USA  
(F) ZIP: 60610

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

30

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Martin, Alice O.  
(B) REGISTRATION NUMBER: 35,601  
(C) REFERENCE/DOCKET NUMBER: 8429/3

35

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (312) 321-4200  
(B) TELEFAX: (312) 321-4299

40

## (2) INFORMATION FOR SEQ ID NO:1:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 785 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

55

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

60

CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT 60

CCTCGTGCCG AATTCGGCAC GAGGCTGCCC ACCCTCTGGA CATTACACCA GCCAGGTGGT 120

CTCGTCACCT CAGAGGCTCC GCAGACTCCT GCCCAGGCCA GGA CTGAGGC AAGCCTCAAG 180  
 GCACCTTCTAG GACCTGGCTC TTCTCACCAA GATGAACTCA CTGGTTTCTT GGCAGCTACT 240  
 5 GCTTTTCCTC TGTGCCACCC ACTTTGGGGA GCCATTAGAA AAGGTGGCCT CTGTGGGGAA 300  
 TTCTAGACCC ACAGGCCAGC AGCTAGAATC CCTGGGCCTC CTGGCCCGGG GAGCAGAGCC 360  
 10 TGCCGTGCAC CGAGAGGAAG CCAGCTGCTA CTGCCAGGCT GAGCCGTCGG GGGACCTCGC 420  
 TGTCCCCGCC CCCCAGAGAGC TCCGGGAGCC GCCAGCAGCA GGGCCTGTCC GCCCCCACA 480  
 GCGCCAGAT CCCCACCC CAGGGCGCGG TGCTGGTGCA GCGGGAGAAG GACCTGCCGA 540  
 15 ACTACAAC TGAACTCCTT GGCCTGCGCT TCGGCAAGCG GGAGGCGGCA CCAGGGAACC 600  
 ACGGCAGAAG CGCTGGGCGG GGCTGGGGCG CAGGTGCGGG GCAGTGAAC TCAGACCCCA 660  
 20 AAGGAGTCAG AGCATGCGGG GCGGGGCGG GGTGGGGGGG ACGTAGGGCT AAGGGAGGGG 720  
 GCGCTGGAGC TTCCAACCC AGGCAATAAA AGAAATGTTG CGTAACTCAA AAAAAAAAAA 780  
 AAAAA 785

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 164 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asn Ser Leu Val Ser Trp Gln Leu Leu Leu Phe Leu Cys Ala Thr  
 1 5 10 15

His Phe Gly Glu Pro Leu Glu Lys Val Ala Ser Val Gly Asn Ser Arg  
 20 25 30

Pro Thr Gly Gln Gln Leu Glu Ser Leu Gly Leu Leu Ala Arg Gly Ala  
 35 40 45

Glu Pro Ala Val His Arg Glu Glu Ala Ser Cys Tyr Cys Gln Ala Glu  
 50 55 60

Pro Ser Gly Asp Leu Ala Val Pro Ala Pro Arg Glu Leu Arg Glu Pro  
 65 70 75 80

Pro Ala Ala Gly Pro Val Arg Pro Pro Gln Pro Pro Asp Pro Arg Thr  
 85 90 95

Pro Gly Arg Gly Ala Gly Ala Ala Gly Glu Gly Pro Ala Glu Leu Gln  
                             100                            105                            110

5 Leu Glu Leu Leu Arg Pro Ala Leu Arg Gln Ala Gly Gly Gly Thr Arg  
                             115                            120                            125

Glu Pro Arg Gln Lys Arg Trp Ala Gly Leu Gly Arg Arg Cys Gly Ala  
                             130                            135                            140

10 Val Asn Phe Arg Pro Gln Arg Ser Gln Ser Met Arg Gly Gly Gly Gly  
                             145                            150                            155                            160

Val Gly Gly Thr

15

## (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 78 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: peptide

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Asn Ser Leu Val Ser Trp Gln Leu Leu Leu Phe Leu Cys Ala Thr  
 1                            5                            10                            15

His Phe Gly Glu Pro Leu Glu Lys Val Ala Ser Val Gly Asn Ser Arg  
                             20                            25                            30

Pro Thr Gly Gln Gln Leu Glu Ser Leu Gly Leu Leu Ala Arg Gly Ala  
                             35                            40                            45

Glu Pro Ala Val His Arg Glu Glu Ala Ser Cys Tyr Cys Gln Ala Glu  
                             50                            55                            60

Pro Ser Gly Asp Leu Ala Val Pro Ala Pro Arg Glu Leu Arg  
                             65                            70                            75

45

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 86 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: peptide

55

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Pro Pro Ala Ala Gly Pro Val Arg Pro Pro Gln Pro Pro Asp Pro  
 1                            5                            10                            15

Arg Thr Pro Gly Arg Gly Ala Gly Ala Ala Gly Glu Gly Pro Ala Glu  
                             20                            25                            30

60



Leu Gln Leu Glu Leu Leu Arg Pro Ala Leu Arg Gln Ala Gly Gly Gly  
           35                                  40                                  45  
 Thr Arg Glu Pro Arg Gln Lys Arg Trp Ala Gly Leu Gly Arg Arg Cys  
 5                  50                                  55                                  60  
 Gly Ala Val Asn Phe Arg Pro Gln Arg Ser Gln Ser Met Arg Gly Gly  
           65                                  70                                  75                                  80  
 10 Gly Gly Val Gly Gly Thr  
                                   85

## (2) INFORMATION FOR SEQ ID NO:5:

15 (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 22 amino acids  
       (B) TYPE: amino acid  
       (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

25 Leu Arg Glu Pro Pro Ala Ala Gly Pro Val Arg Pro Pro Gln Pro Pro  
       1                                  5                                  10                                  15  
 30 Asp Pro Arg Thr Pro Gly  
                                   20

## (2) INFORMATION FOR SEQ ID NO:6:

35 (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 3 amino acids  
       (B) TYPE: amino acid  
       (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

45 Ser Met Arg  
       1

## (2) INFORMATION FOR SEQ ID NO:7:

50 (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 234 base pairs  
       (B) TYPE: nucleic acid  
       (C) STRANDEDNESS: single  
       (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGAACTCAC TGGTTTCTTG GCAGCTACTG CTTTTCCTCT GTGCCACCCA CTTTGGGGAG

60

CCATTAGAAA AGGTGGCCTC TGTGGGGAAT TCTAGACCCA CAGGCCAGCA GCTAGAATCC 120  
 CTGGGCCTCC TGGCCCGGGG AGCAGAGCCT GCCGTGCACC GAGAGGAAGC CAGCTGCTAC 180  
 5 TGCCAGGCTG AGCCGTCGGG GGACCTCGCT GTCCCCGCCC CCCGAGAGCT CCGG 234

## (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
 10 (A) LENGTH: 258 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

20 GAGCCGCCAG CAGCAGGGCC TGTCCGCCCC CCACAGCCGC CAGATCCCCG CACCCCAGGG 60  
 CGCGGTGCTG GTGCAGCGGG AGAAGGACCT GCCGAACTAC AACTGGAACCT CCTTCGGCCT 120  
 25 GCGCTTCGGC AAGCGGGAGG CGGCACCAGG GAACCACGGC AGAAGCGCTG GCGGGGGCTG 180  
 GGGCGCAGGT GCGGGGCACT GAACTTCAGA CCCCAAAGGA GTCAGAGCAT GCGGGGCGGG 240  
 GCGGGGGTGG GGGGGACG 258

30 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
 35 (A) LENGTH: 66 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

45 CTCCGGGAGC CGCCAGCAGC AGGGCCTGTC CGCCCCCAC AGCCGCCAGA TCCCCGCACC 60  
 CCAGGG 66

## (2) INFORMATION FOR SEQ ID NO:10:

50 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

60

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCATGCGG

**WE CLAIM:**

1. A cDNA molecule having a nucleotide sequence in accordance with SEQ ID NO: 1:

```

5  CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT 60
   CCTCGTGCCG AATTCGGCAC GAGGCTGCCC ACCCTCTGGA CATTACACCA GCCAGGTGGT 120
   CTCGTCACCT CAGAGGCTCC GCAGACTCCT GCCCAGGCCA GGACTGAGGC AAGCCTCAAG 180
   GCACTTCTAG GACCTGGCTC TTCTCACCAA GATGAACTCA CTGGTTTCTT GGCAGCTACT 240
   GCTTTTCCTC TGTGCCACCC ACTTTGGGGA GCCATTAGAA AAGGTGGCCT CTGTGGGGAA 300
10 TTCTAGACCC ACAGGCCAGC AGCTAGAATC CCTGGGCCTC CTGGCCCGGG GAGCAGAGCC 360
   TGCCGTGCAC CGAGAGGAAG CCAGCTGCTA CTGCCAGGCT GAGCCGTCGG GGGACCTCGC 420
   TGTCCCCGCC CCCCAGAGAG TCCGGGAGCC GCCAGCAGCA GGGCCTGTCC GCCCCCACA 480
   GCCGCCAGAT CCCCACCCC CAGGGCGCGG TGCTGGTGCA GCGGGAGAAG GACCTGCCGA 540
   ACTACAACTG GAACTCCTTC GGCCTGCGCT TCGGCAAGCG GGAGGCGGCA CCAGGGAACC 600
15 ACGGCAGAAG CGCTGGGCGG GGCTGGGGCG CAGGTGCGGG GCAGTGAAC TCAGACCCCA 660
   AAGGAGTCAG AGCATGCGGG GCGGGGGCGG GGTGGGGGGG ACGTAGGGCT AAGGGAGGGG 720
   GCGCTGGAGC TTCCAACCCG AGGCAATAAA AGAAATGTTG CGTAACTCAA AAAAAAAAAA 780
   AAAAAA 785.

```

20 2. A protein having an amino acid sequence encoded by the cDNA molecule of claim 1.

3. A protein having an amino acid sequence in accordance with SEQ ID NO. 2:

```

25 Met Asn Ser Leu Val Ser Trp Gln Leu Leu Leu Phe Leu Cys Ala Thr
   1             5             10             15

   His Phe Gly Glu Pro Leu Glu Lys Val Ala Ser Val Gly Asn Ser Arg
30             20             25             30

   Pro Thr Gly Gln Gln Leu Glu Ser Leu Gly Leu Leu Ala Arg Gly Ala
             35             40             45

35 Glu Pro Ala Val His Arg Glu Glu Ala Ser Cys Tyr Cys Gln Ala Glu
   50             55             60

   Pro Ser Gly Asp Leu Ala Val Pro Ala Pro Arg Glu Leu Arg Glu Pro
40 65             70             75             80

   Pro Ala Ala Gly Pro Val Arg Pro Pro Gln Pro Pro Asp Pro Arg Thr
             85             90             95

```

Pro Gly Arg Gly Ala Gly Ala Ala Gly Glu Gly Pro Ala Glu Leu Gln  
 100 105 110

5 Leu Glu Leu Leu Arg Pro Ala Leu Arg Gln Ala Gly Gly Gly Thr Arg  
 115 120 125

Glu Pro Arg Gln Lys Arg Trp Ala Gly Leu Gly Arg Arg Cys Gly Ala  
 130 135 140

10 Val Asn Phe Arg Pro Gln Arg Ser Gln Ser Met Arg Gly Gly Gly Gly  
 145 150 155 160

Val Gly Gly Thr.

15

4. A cDNA molecule having a nucleotide sequence in  
 accordance with SEQ ID NO: 7:

ATGAACTCAC TGGTTTCTTG GCAGCTACTG CTTTTCCTCT GTGCCACCCA CTTTGGGGAG 60  
 CCATTAGAAA AGGTGGCCTC TGTGGGGAAT TCTAGACCCA CAGGCCAGCA GCTAGAATCC 120  
 20 CTGGGCCTCC TGGCCCGGGG AGCAGAGCCT GCCGTGCACC GAGAGGAAGC CAGCTGCTAC 180  
 TGCCAGGCTG AGCCGTCGGG GGACCTCGCT GTCCCCGCC CCCGAGAGCT CCGG 234.

5. A cDNA molecule having a nucleotide sequence in  
 25 accordance with SEQ ID NO: 8:

GAGCCGCCAG CAGCAGGGCC TGTCCGCCCC CCACAGCCGC CAGATCCCCG CACCCCAGGG 60  
 CGCGGTGCTG GTGCAGCGGG AGAAGGACCT GCCGAACCTAC AACTGGAACCT CTTTCGGCCT 120  
 GCGCTTCGGC AAGCGGGAGG CGGCACCAGG GAACCACGGC AGAAGCGCTG GCGGGGGCTG 180  
 GGGCGCAGGT GCGGGGCAGT GAACTTCAGA CCCCAGAGGA GTCAGAGCAT GCGGGGCGGG 240  
 30 GGCGGGGTGG GGGGGACG 258.

6. A cDNA molecule having a nucleotide sequence in  
 accordance with SEQ ID NO: 9:

35 CTCCGGGAGC CGCCAGCAGC AGGGCCTGTC CGCCCCCAC AGCCGCCAGA TCCCCGCACC 60  
 CCAGGG 66.

7. A cDNA molecule having a nucleotide sequence in  
 accordance with SEQ ID NO: 10:

40 AGCATGCGG 9.

8. A polypeptide having an amino acid sequence in accordance with SEQ ID NO: 3:

5  
Met Asn Ser Leu Val Ser Trp Gln Leu Leu Leu Phe Leu Cys Ala Thr  
1 5 10 15  
His Phe Gly Glu Pro Leu Glu Lys Val Ala Ser Val Gly Asn Ser Arg  
10 20 25 30  
Pro Thr Gly Gln Gln Leu Glu Ser Leu Gly Leu Leu Ala Arg Gly Ala  
35 40 45  
15 Glu Pro Ala Val His Arg Glu Glu Ala Ser Cys Tyr Cys Gln Ala Glu  
50 55 60  
Pro Ser Gly Asp Leu Ala Val Pro Ala Pro Arg Glu Leu Arg.  
65 70 75

20

9. A polypeptide having an amino acid sequence in accordance with SEQ ID NO: 4:

25 Glu Pro Pro Ala Ala Gly Pro Val Arg Pro Pro Gln Pro Pro Asp Pro  
1 5 10 15  
Arg Thr Pro Gly Arg Gly Ala Gly Ala Ala Gly Glu Gly Pro Ala Glu  
20 25 30  
30 Leu Gln Leu Glu Leu Leu Arg Pro Ala Leu Arg Gln Ala Gly Gly Gly  
35 40 45  
Thr Arg Glu Pro Arg Gln Lys Arg Trp Ala Gly Leu Gly Arg Arg Cys  
50 55 60  
35 Gly Ala Val Asn Phe Arg Pro Gln Arg Ser Gln Ser Met Arg Gly Gly  
65 70 75 80  
Gly Gly Val Gly Gly Thr.  
40 85

10. A polypeptide having an amino acid sequence in  
5 accordance with SEQ ID NO: 5:

Leu Arg Glu Pro Pro Ala Ala Gly Pro Val Arg Pro Pro Gln Pro Pro  
1 5 10 15  
Asp Pro Arg Thr Pro Gly.  
20

10

11. A polypeptide having an amino acid sequence in  
accordance with SEQ ID NO: 6:

Ser Met Arg  
1

- 15 and flanking amino acid sequences that determine binding  
specificity.

12. A recombinant expression system comprising a  
suitable regulatory system and the nucleotide sequence of  
20 claim 1 or fragments or truncations thereof.

13. A marker for distinguishing metastatic from  
non-metastatic cells, said marker comprising a labeled  
nucleotide sequence in accordance with SEQ ID NO: 1, or  
25 fragments or truncations thereof.

14. A marker for distinguishing metastatic from  
non-metastatic cells, said marker comprising a labeled  
amino acid sequence in accordance with SEQ ID NO: 2 or  
30 fragment or truncations thereof.

15. A method for staging the oncogenetic process in  
a subject, said method comprising determining the level  
of expression of a polypeptide having an amino acid  
35 sequence in accordance with SEQ ID NO: 2 in a biological  
sample obtained from the subject.

16. A method for planning therapy for a cancer in a  
subject, said method comprising determining whether the

cDNA of claim 1 or a fragment or truncation thereof is expressed in a sample of cancer cells from the subject, and comparing the level of expression to a set of standards for stages of the cancer.

5

17. The method of claim 16, where the cancer is cutaneous melanoma.

18. A method for diagnosing the presence of a metastasizing cancer in a subject, said method comprising determining whether a polypeptide having an amino acid sequence in accordance with SEQ ID NO: 2 or a fragment or truncation thereof, is present in a biological sample from the subject at levels that indicate metastasis.

15

19. A method for introducing a metastasis suppressor gene into a subject, said method comprising obtaining an expression system for a gene having a nucleotide sequence in accordance with SEQ ID NO: 1 or a fragment or truncation thereof, and transferring the expression system into the subject.

20

20. A method for treating a cancer to prevent metastasis, said method comprising administering to a subject with cancer an effective amount of a polypeptide having an amino acid sequence in accordance with SEQ ID NO: 2, or a fragment or truncation thereof.

25

21. A pharmaceutical composition comprising a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID. NOS: 2, 3, 4, 5 and 6, in a suitable diluent.

30

```
CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT 60
CCTCGTGCCG AATTCGGCAC GAGGCTGCCC ACCCTCTGGA CATTCAACCA GCCAGGTGGT 120
CTCGTCACCT CAGAGGCTCC GCAGACTCCT GCCCAGGCCA GGA CTGAGGC AAGCCTCAAG 180
GCACTTCTAG GACCTGGCTC TTCTCACCAA GATGAACTCA CTGGTTTCTT GGCAGCTACT 240
GCTTTTCCTC TGTGCCACCC ACTTTGGGGA GCCATTAGAA AAGGTGGCCT CTGTGGGGAA 300
TTCTAGACCC ACAGGCCAGC AGCTAGAATC CCTGGGCCTC CTGGCCCGGG GAGCAGAGCC 360
TGCCGTGCAC CGAGAGGAAG CCAGCTGCTA CTGCCAGGCT GAGCCGTCGG GGGACCTCGC 420
TGTCCCCGCC CCCCAGAGC TCCGGGAGCC GCCAGCAGCA GGGCCTGTCC GGGGGGACCA 480
GCCGCCAGAT CCCCACCCC CAGGGCGCGG TGCTGGTGCA GCGGGAGAAG GACCTGCCGA 540
ACTACAACCTG GAACTCCTTC GGCCTGCGCT TCGGCAAGCG GGAGGCGGCA CCAGGGAACC 600
ACGGCAGAAG CGCTGGGCGG GGCTGGGGCG CAGGTGCGGG GCAGTGAAC TACAGACCCA 660
AAGGAGTCAG AGCATGCGGG GCGGGGGCGG GGTGGGGGGG ACGTAGGGCT AAGGGAGGGG 720
GCGCTGGAGC TTCCAACCCG AGGCAATAAA AGAAATGTTG CGTAACTCAA AAAAAAAAAA 780
AAAAA 785
```

FIG. 1



Met	Asn	Ser	Leu	Val	Ser	Trp	Gln	Leu	Leu	Leu	Phe	Leu	Cys	Ala	Thr
1				5					10					15	
His	Phe	Gly	Glu	Pro	Leu	Glu	Lys	Val	Ala	Ser	Val	Gly	Asn	Ser	Arg
		20					25						30		
Pro	Thr	Gly	Gln	Gln	Leu	Glu	Ser	Leu	Gly	Leu	Leu	Ala	Arg	Gly	Ala
		35					40						45		
Glu	Pro	Ala	Val	His	Arg	Glu	Glu	Ala	Ser	Cys	Tyr	Cys	Gln	Ala	Glu
	50					55					60				
Pro	Ser	Gly	Asp	Leu	Ala	Val	Pro	Ala	Pro	Arg	Glu	Leu	Arg	Glu	Pro
65				70						75				80	
Pro	Ala	Ala	Gly	Pro	Val	Arg	Pro	Pro	Gln	Pro	Pro	Asp	Pro	Arg	Thr
			85						90					95	
Pro	Gly	Arg	Gly	Ala	Gly	Ala	Ala	Gly	Glu	Gly	Pro	Ala	Glu	Leu	Gln
			100					105					110		
Leu	Glu	Leu	Leu	Arg	Pro	Ala	Leu	Arg	Gln	Ala	Gly	Gly	Gly	Thr	Arg
		115					120						125		
Glu	Pro	Arg	Gln	Lys	Arg	Trp	Ala	Gly	Leu	Gly	Arg	Arg	Cys	Gly	Ala
	130					135					140				
Val	Asn	Phe	Arg	Pro	Gln	Arg	Ser	Gln	Ser	Met	Arg	Gly	Gly	Gly	Gly
145					150					155				160	
Val	Gly	Gly	Thr												

FIG. 2

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/16414

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 536/23.1; 530/300, 350; 435/4, 320.1; 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/300, 350; 435/4, 320.1; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	LEE et al. Use of differential display and subtractive hybridization to identify highly expressed genes in metastasis-suppressed chromosome 6/human melanoma hybrids. Proceedings of the American Association for Cancer Research. March 1996, Vol. 37, page 76, abstract no. 530, see entire abstract.	1-21
Y, P	LEE et al. Cloning of a novel gene, KiSS-1, which is responsible for metastasis suppression in chromosome 6/human melanoma hybrid cells. Proceedings of the American Association for Cancer Research. March 1996, Vol. 37, page 76, abstract no. 531, see entire abstract.	1-21
Y	WO 95/23814 A1 (NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE) 08 September 1995, see entire document.	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 DECEMBER 1996

Date of mailing of the international search report

17 JAN 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/16414

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C07H 21/04; C07K 5/00, 14/00; C12N 15/63; A61K 38/00, 48/00; C12Q 1/68

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